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We are testing the hypothesis that a model parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells *in vitro* and *in vivo*. We have developed a novel strategy to establish the protozoan parasite *T. gondii* as the next generation vector for breast cancer gene therapy. The significant innovative aspect of this approach is the promise of this strategy to deliver a novel vector for breast cancer gene therapy that is superior to the current vectors under current development and refinement. The primary purpose and scope of this IDEA award project is to experimentally examine approaches to target the *Toxoplasma gondii* parasite gene therapy vector to breast cancer tissue using *in vitro* and *in vivo* models. In this reporting period we have found that both the cytosine deaminase (CD) and thymidine kinase (TK) markers expressed in *T. gondii* produce a bystander killing effect on cells *in vitro*. Using trifunctional enzyme expression plasmids we have co-expressed both the TK and CD markers in transgenic *T. gondii*. A newly developed and highly attenuated (avirulent) auxotroph mutant of *T. gondii* will provide an improved vector for targeting breast cancer gene therapy.

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Introduction

We are testing the hypothesis that a model parasite gene therapy vector can be genetically altered to safely, specifically and effectively target breast cancer cells *in vitro* and *in vivo*. We have developed a novel strategy to establish the protozoan parasite *T. gondii* as the next generation vector for breast cancer gene therapy. The significant innovative aspect of this approach is the promise of this strategy to deliver a novel vector for breast cancer gene therapy that is superior to the current vectors under current development and refinement. The primary purpose and scope of this IDEA award project is to experimentally examine approaches to target the *Toxoplasma gondii* parasite gene therapy vector to breast cancer tissue using *in vitro* and *in vivo* models.

Body

The statement of work is reiterated below for reporting period:

Task 1: Months 1-6: Construct and document CD-TK fusion for co-expression of suicide genes. Compare fusion protein to currently established single gene tools for expressing CD and TK.

Task 2: Months 7-10: Develop bispecific antibody for targeting HER2/neu.

Research Accomplishments associated with each task:

Task 1: Months 1-6: Construct and document CD-TK fusion for co-expression of suicide genes. Compare fusion protein to currently established single gene tools for expressing CD and TK.

We have documented the expected bystander effect for transgenic *T. gondii* expressing thymidine kinase (TK). Assessment by the DEAD red cell assay we found that infecting 10% of the cell population with TK expressing *T. gondii*, followed by treatment of the cell culture with 5 μ M ganciclovir resulted in greater than 90% killing of the uninfected cell population after 24 h incubation.

We have attempted several types of plasmid constructs (Figure 1, appendix) for developing a cytosine deaminase (CD) and thymidine kinase (TK) gene fusion construct that will stably express both enzyme activities in *T. gondii*. Direct fusions of CD-TK or TK-CD failed to yield expression of both enzyme activities. These experiments were complicated by the absence of a positive selectable marker in transfecting these constructs into *T. gondii*. Thus we are unsure if the difficulty is the lack of selection, the failure of the construct to express both enzyme activities, the overall efficiency, or a combination of these potential problems. It should be noted that in theory, it should be possible to co-express separate single TK and single CD gene constructs in the same transgenic parasite. However, in the absence of a selection procedure it would require a significant effort to score, screen and empirically obtain clones with high sensitivity to the two prodrugs in use.

In a second approach we attempted to construct quadfunctional DHFR-TK-CD-TS plasmids based on our success at expressing the trifunctional DHFR-TK-TS, or DHFR-CD-TS enzymes. This approach did work....albeit with limited success due to the apparent low expression of the markers from this plasmid construct. To circumvent these problems we have solved the problem posed by

Task 1 by co-transfected our successful trifunctional DHFR-TK-TS and DHFR-CD-TS plasmids into *T. gondii*. Selection in pyrimethamine produced transgenic parasites and upon subcloning we found that approximately 20% of clones had acquired sensitivity to both 5 μ M 5-fluorocytosine and 5 μ M ganciclovir. These transgenic clones are currently under further evaluation to document allele numbers and to identify clones which have the higher sensitivities to the prodrugs 5-fluorocytosine and ganciclovir.

Related to the task of constructing transgenic *T. gondii* expressing both TK and CD is the development of a "safe" or "safer" strain of *T. gondii* which could be more appropriately used and controlled in the in vitro and in vivo targeting studies. We wish to report that in independent work our laboratory has recently produced a pyrimidine-requiring auxotroph mutant of *T. gondii* that has very attractive properties for its potential use in the breast cancer targeting studies. This auxotrophic mutant is extremely attenuated in both immune competent and immunocompromised mice [it is the first *T. gondii* mutant that will not harm a series of severely immunocompromised mice]. The mutant invades host cells normally and will express proteins for several days; however, this mutant does not appear to replicate in vitro or in vivo in the absence of pyrimidine supplementation. For these reasons we logically plan to extend task 1 to include the development of the expression of TK and CD markers, at useful levels, in the pyrimidine auxotroph mutant strain of *T. gondii*.

Task 2: Months 7-10: Develop bispecific antibody for targeting HER2/neu.

We have made progress on task 2, however, for several reasons task 2 is not yet fully completed. We have produced approximately 20 mg of anti-HER-2/neu Mab 520C9 and the resulting Fab' which is required for the construction of the bispecific targeting antibody. However, the antibody (Mab6A8) we obtained to the major surface antigen of *T. gondii*, the P30 antigen, does not appear to have sufficient avidity/affinity to warrant its use in a bispecific construct. The Mab6A8 was found to be on a relative basis have a 50-fold less avidity/affinity for binding the *T. gondii* P30 surface antigen than a polyclonal rabbit (Ware and Kasper 1987). For these reasons we are currently scheduling the production of a higher affinity/avidity anti-P30 polyclonal serum that can produce an improved bispecific antibody. For the construction of the bispecific we have recently obtained the assistance/consulting of an expert in this area, Dr. Robert Graziano.

Problems in accomplishing tasks:

While we have made respectable progress in accomplishing the intial tasks 1 and tasks 2 we have also experienced some difficulties in this work. The scientific based difficulties were mentioned above. In addition to these experimental problems we had the unfortunate circumstance that a student who was working on this project left the lab and PhD. Program in September of 1999. After this, my initial plan was to recruit 1 or 2 new students to this project and to train them appropriately, however students are not "guaranteed" in my Ph.D. program and none became available in Fall and Winter 1999/2000. For these reasons I attempted to recruit a new Research Technician to manage this project and was very fortunate, particularly in the difficult current recruiting climate, to hire in April 2000 an intelligent, experienced and qualified individual who is very excited about working on this breast cancer targeting. This recruitment should assure current and future progress on the project tasks.

Key Research Accomplishments

- Co-expression of cytosine deaminase and thymidine kinase in *T. gondii*
- TK transgenic *T. gondii* exhibit the bystander effect with ganciclovir treatment
- An highly attenuated and avirulent strain of *T. gondii* is now available for implementation in breast cancer targeting studies

Reportable Outcomes

No outcomes are yet reportable

Conclusions

Both the thymidine kinase and cytosine deaminase genetic markers have been expressed in transgenic *T. gondii* and express the "bystander" effect. That is, that neighboring cells to those expressing CD or TK can be killed by the toxic products formed following treatment with 5-fluorocytosine or ganciclovir, respectively. Transgenic *T. gondii* have been obtained that co-express the TK and CD transgenes. The development of a targeting strategy employing bispecific antibody or stable genetic equivalents is being developed to target the TK and CD activities to breast cancer tissue(s) in vitro and in vivo. A mutant parasite recently developed in my laboratory is a highly attenuated and avirulent strain of *T. gondii* and a pyrimidine-requiring auxotroph mutant. The development of this avirulent mutant has importance for the current project in that this mutant strain will provide a "safer" vector for use in the targeting studies, as well as a parasite that may be more useful in a variety of ways. While this is not as crucial for the near term in vitro studies, the availability of this attenuated mutant is important when considering the in vivo studies. For example, because of the availability of this mutant in vivo studies can now be done in immunocompromised mice, or normal mice. In addition, studies at direct inoculation of in vivo mice tumors is now possible using this avirulent mutant. Further, as the mutant parasite does not appear to replicate in vivo, this approach should provide an excellent avenue for more precise targeting, as well as more quantitative studies that control the exact parasite does(s). For these reasons we believe it is warranted to extend task 1 to develop TK and CD expressing parasites in the mutant strain that is the avirulent pyrimidine auxotroph.

References

1. Ware, P.L. and L. H. Kasper. (1987) Strain specific antigens of *Toxoplasma gondii*. *Inf. Immunity* 55: 778.

Appendices

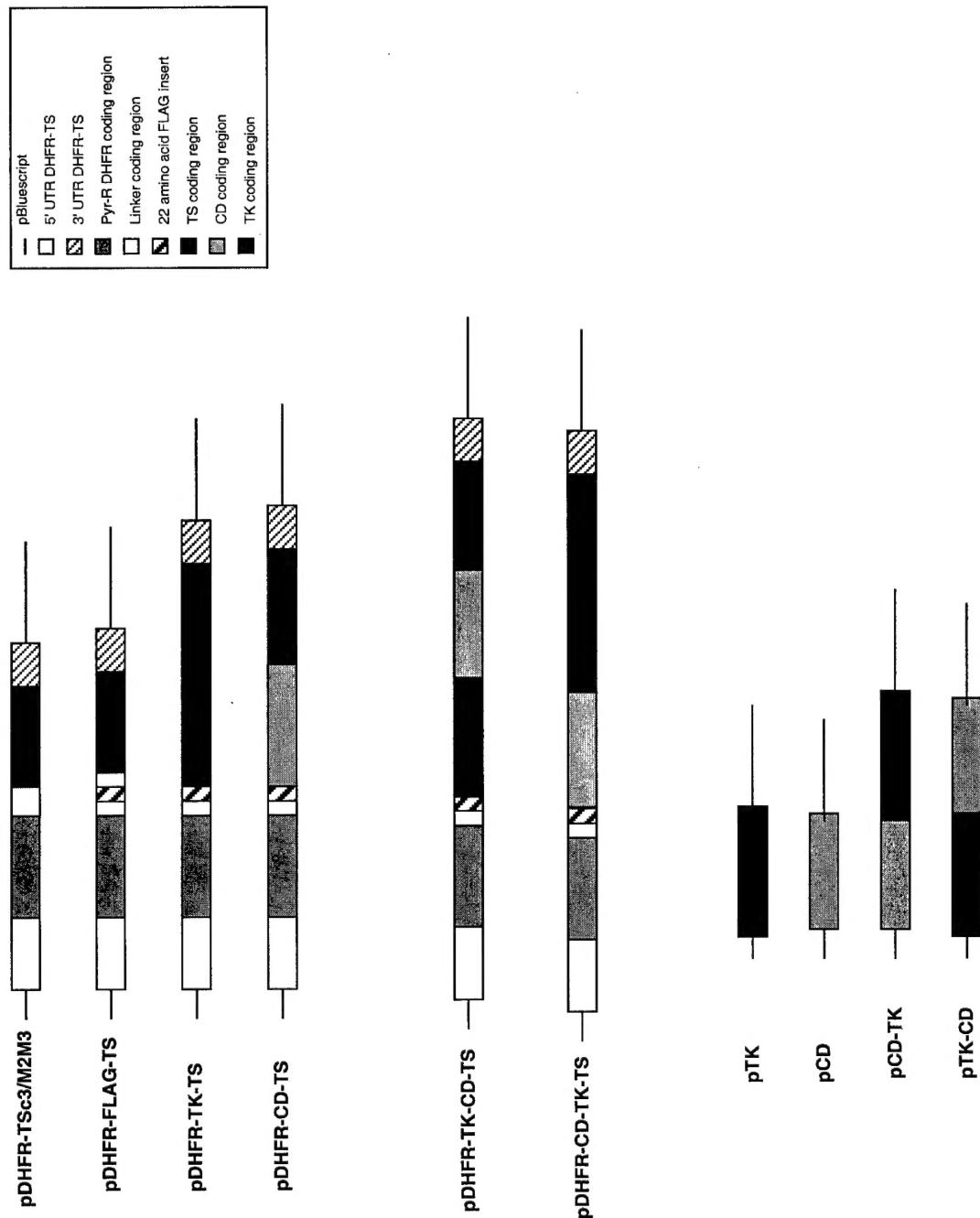


Figure 1. Monofunctional, bifunctional, trifunctional and quad functional enzyme plasmid constructs for expression of thymidine kinase and cytosine deaminase in transgenic *Toxoplasma gondii*.